REMARKS

I. Claim Status

Claims 16, 17 and 21-28 were pending in the present application. Claims 17, 21, and 25-28 are cancelled herewith without prejudice or disclaimer. Upon entry of the present amendment, claims 16, 22-24, and 29-34 will be pending in this application.

II. Amendments to the Claims

Claim 16 has been amended. The amendment to claim 16 emphasizes the inventive features of the claimed antibodies as binding to living MCF-7 or 3T3 cells expressing human or murine BCRP on their surface, and that antibodies of the present invention do not bind to MCF-7 cells that do not express human or murine BCRP on their surface. Support for this claim may be found in paragraphs 74-77 of the published specification.

New claims 29 and 30 are directed to antibodies operably attached to a detectable label and immunodetection kits. Support for these claims may be found in paragraphs 73-81 of the published specification.

New claim 31 is directed to an isolated antibody isolated according to the steps involving immunizing an animal with 3T3 cells that express human or murine BCRP in its natural conformation on the cell surface and selecting and isolating a desired hybridoma. New claim 33 claims an antibody isolated from a hybridoma generated in accordance with the invention, e.g., by steps similar to those recited in claim 31. Support for these claims may be found in paragraphs 74-77 of the published specification. New claims 32 and 34, which depend from claims 31 and 33, respectively, recite that the BCRP is human BCRP and that the immunized animal is a mouse. Applicants assert that claims 31-34 should be examined together with the presently pending claims, since they encompass an antibody product with the same binding properties as that of claim 16. Examining claims 31-34 in the present case would not present any additional searching burden for the Examiner.

Applicants assert that no new matter has been introduced in the present amendments.

III. Telephonic Interview Summary

Applicants thank the Examiner for discussing the present issues in a telephonic interview on November 29, 2005. Applicants note that in view of the arguments presented below and the relevant case law cited therein, the Examiner has indicated the rejections under 35 U.S.C. §112, first paragraph, will be withdrawn. Applicants present the below arguments in Section IV for the record and to be fully responsive to the office action of July 28, 2005. Additionally, the 35 U.S.C. §103 rejection of claims 16, 17, 21-28 over Ross *et al.* (U.S. Patent No. 6,313,277) in view of Mechetner *et al.* (U.S. Patent No. 5,994,088) were discussed. The Examiner has requested evidence that using the antigen taught by Ross, and the method taught by Ross and Mechetner, that these antibodies would lack the functions of Applicants' antibodies.

The combination of Ross and Mechetner does not teach the inventive antibodies. In support of this view, Applicants provide in Section V below, data describing the functional distinctions of exemplary inventive antibody 5D3 over antibodies BXP-21 and BXP-34. To summarize, neither BXP-21 nor BXP-34 was capable of binding specifically to a native human or murine ABCG2/BCRP conformation expressed on living cells, and these failures highlight the distinguishing features of the inventive antibodies exemplified by 5D3. Additionally, antibody 5D3 fails to react with ABCG2 on immunoblots, illustrating that 5D3 does not bind to denatured, purified ABCG2. Thus, the antibodies of the present invention are clearly distinguished from the antibodies of the prior art in view of this data and in view of the complete arguments provided below.

IV. Rejections under 35 U.S.C. §112, first paragraph

Claims 16, 17, 21-28 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors at the time the application was

filed, had possession of the claimed genus of invention, i.e., for allegedly lacking written description.

In view of the amendments to claim 16, Applicants assert that this rejection is overcome. The specification provides adequate written description for the claimed isolated antibodies that bind to an extracellular portion of a Breast Cancer Resistance Protein (BCRP) that is either a human or murine BCRP; wherein the extracellular portion of the BCRP is in its natural conformation; wherein the antibodies bind to living MCF-7 or 3T3 cells expressing BCRP on their surface; and wherein the antibodies fail to bind to living MCF-7 cells that do not express BCRP (as described in paragraphs 74-77 of the published specification).

Applicants point to the recent Federal Circuit case of *Noelle v. Lederman,* 355 F.3d 1343, 1349 (Fed. Cir. 2004), in which the court recognized that an adequate description of a claimed class of antibodies depends not on a description of the physical structure of any particular antibody within the class, but on a description which allows one of skill in the art to produce such an antibody. The court in *Noelle v. Lederman* instructed that "as long as an applicant has disclosed a 'fully characterized antigen' either by its structure, formula, chemical name, or physical properties, or by depositing the protein in a public depository, the applicant can then claim an antibody by its binding affinity to that described antigen." In Noelle, such a description took the form of a biochemical description (including molecular weight; cell type and cellular location; and being immunologically recognized by a specific deposited antibody) of the fully characterized antigen, which the claimed antibody specifically recognized. In the present case this description takes the form of critical properties of the claimed antibodies with respect to their ability to bind to living MCF-7 or 3T3 cells expressing BCRP on their surface and inability to bind to living MCF-7 cells that do not express BCRP. These are the properties disclosed by the present invention which allow the claimed antibodies to be produced. The full structure (sequence) of the human and murine BCRP sequences were known, as indicated by reference to the specific amino acid sequences at paragraph 25 of the published specification. By virtue of the disclosed fully characterized human and murine BCRP antigens, and recitation of the native structure of the antigens as recognized by the claimed antibodies, the presently claimed antibodies meet the requirements of 35 U.S.C. §112,

first paragraph, and the rejection should be withdrawn. See also *Capon v. Esshar v. Dudas*, 418 F.3d 1349 (Fed. Cir. 2005) (holding that the Board of Patent Appeals and Interferences "erred in ruling that §112 imposes a *per se* rule requiring recitation in the specification of the nucleotide sequence of claimed DNA, when that sequence is already known in the field.").

Claims 16, 17, 21-28 stand rejected under 35 U.S.C. §112, first paragraph, because according to the Examiner, the specification, while being enabling for using anti-BCRP antibodies 5D3, 7A3, 1C5 and 8C2, does not reasonably provide enablement for making and using the claimed genus of anti-BCRP antibodies. In view of the amendments to claim 16, Applicants assert that this rejection is overcome. The specification enables making and using isolated antibodies that bind to an extracellular portion of a Breast Cancer Resistance Protein (BCRP) that is either a human or murine BCRP; wherein the extracellular portion of the BCRP is in its natural conformation; wherein the antibodies bind to living MCF-7 or 3T3 cells expressing BCRP on their surface; and wherein the antibodies fail to bind to living MCF-7 cells that do not express BCRP (as described in paragraphs 74-77 of the specification).

Indeed, the Examiner's acknowledgement of four separate examples of species (5D3, 7A3, 1C5, and 8C2) within the scope of claim 16 contradicts the contention that the specification does not enable claim 16. In a well-developed art such as the monoclonal antibody arts, a single exemplary species within the scope of the claims substantiates enablement of the claimed genus (See, *e.g., Hybritech v. Monoclonal Antibodies*[1]*; and In re Wands*[2]).

With regard to the Examiner's characterization of the declaration statements of Dr. Sarkadi, it should be noted that these statements refer to the expectations of the skilled artisan prior to Applicant's filing without the benefit of the knowledge imparted by the present invention. The Examiner has focused on the wrong issue with regard to the antibody production techniques. Prior to the inventors' disclosure, no one had successfully reported the production of antibodies that

---

[1] *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367,1384 (Fed. Cir. 1986) (methods for obtaining and screening monoclonal antibodies were well known in 1980).

[2] *In re Wands*, 858 F.2d at 737 (Fed. Cir. 1988) (describing the focus of a proper enablement inquiry as one that looks at the evidence as a whole and held that a specification was enabling for claims where among other factors "all of the methods needed to practice the invention were well known.") Applicants note that *Wands* concerned enablement of a monoclonal antibody and is directly on point.

would bind specifically to a native human or murine BCRP conformation as expressed on living cells. This is not surprising since skilled artisans would have been inclined not to try to accomplish this using available techniques given the prevailing sentiment that such techniques would NOT work for this purpose, as the Sarkadi declaration explains (at page 2). The present inventors overcame these failures and successfully made the desired antibody. Once the discovery disclosed in this application overcame the conventional wisdom that the available techniques would not work to generate an antibody to the natural conformation of BCRP, making additional antibodies of the type claimed simply becomes a matter of routine repetition of the disclosed process. It is from this perspective that Dr. Sorrentino's statements from the February 2005 declaration are relevant and consistent with the inventive antibodies. By repeating the procedures described and enabled by the inventors, antibodies encompassed by amended claim 16 were generated, including: 5D3, 7A3, 1C5, and 8C2. These antibodies have all been identified by the Examiner as meeting the enablement provision of 35 U.S.C. §112, first paragraph (see Office Action pg. 6).

Claims 21 and 25 have been cancelled. Thus, the Examiner's rejection with respect to these claims is moot.

Claims 27 and 28 have been cancelled.

With regard to claims 23 and 24 directed to chimeric and humanized antibodies, respectively, Applicants assert that making chimeric and humanized antibodies based upon the inventive antibodies is well within the ability of one skilled in the art. Techniques for generating chimeric and humanized antibodies are cited and incorporated by reference at paragraphs 70-73 of the published specification (citing the teachings of Morrison, Neuberger, and Takeda). The dates of these teachings go back to the late 1980's and are well established and routine. Thus, the antibodies of the present invention are enabled, and the rejections under 35 U.S.C. §112, first paragraph are obviated in light of the amendment of claim 16.

V. Rejections under 35 U.S.C. §103

Claims 16, 17, 21-28 stand rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Ross *et al.* (U.S. Patent No. 6,313,277) in view of Mechetner *et al.* (U.S. Patent No. 5,994,088).

In response, Applicants point out that combining Mechetner and Ross would not provide or even suggest the claimed antibodies. Mechetner teaches methods related to generating antibodies to various conformations of Pgp proteins (P-glycoproteins, ABC transporter proteins that function as an efflux pump), including to mutants, to various extracellular conformations, and to substrate-bound conformations. Pgp protein exists as a single contiguous peptide molecule, capable of functioning alone as a transporter. Mechetner is silent regarding BCRP proteins, BCRP conformations, and BCRP antibodies. At best, Mechetner teaches a method relating to producing antibodies to a single peptide chain transporter, and provides no teachings or indications of success for producing antibodies to a functional two-component homodimer or heterodimer transporter.

Ross describes the human BCRP amino acid sequence and its corresponding cDNA. Ross describes antagonists, including immunoglobulins to BCRP that inhibit or modulate the pump function of BCRP. Additionally, Ross contemplates preparing polyclonal antibodies capable of binding to BCRP by immunizing a mammal with a preparation of BCRP or a BCRP derivative. The only mention of monoclonal antibodies in Ross (Column 4, lines 33-60) is a general statement about using monoclonal antibodies to assay for BCRP in a sample and states that such antibodies can be produced by immunizing splenocytes with activated BCRP (citing to the general methods for making antibodies of Kohler et al. (Nature 256:495 (1975); Eur. J. Immunol. 6:511 (1976); Eur. J. Immunol. 6:292 (1976)). Thus, Ross contemplates generating antibodies using purified BCRP protein and is silent regarding generating antibodies to the BCRP protein in its native conformation or to extracellular domains.

Thus, the combined references neither teach nor enable (and thus fail to provide a reasonable expectation of success) generating the claimed antibodies to a natural conformation of BCRP, or expression of recombinant BCRP in host cells suitable for immunization and in other host

cells suitable for screening. The presently claimed antibodies bind to an extracellular portion of a Breast Cancer Resistance Protein (BCRP) that is either a human or murine BCRP; wherein the extracellular portion of the BCRP is in its natural conformation; wherein the antibodies bind to living MCF-7 or 3T3 cells expressing BCRP on their surface; and wherein the antibodies fail to bind to living MCF-7 cells that do not express BCRP. In order to visually highlight the predicted extracellular epitopes of BCRP, Applicants submit Exhibit 1, a diagram of BCRP, showing its predicted secondary structure.

The specificity of an exemplary inventive antibody, 5D3 described in Dr. Sarkadi's Supplemental Declaration of January 17, 2005, was illustrated in experiments described in the pre-publication of Dr. Sarkadi: Özvegy-Laczka *et al.* December 2, 2004; Journal of Biological Chemistry, (a copy of which was submitted with the response of February 22, 2005 and a duplicate copy is submitted herewith). This article has since published as Özvegy-Laczka *et al.*, 2005, *J. Biol. Chem.*; 280: 4219-4227; a copy of which is being submitted concurrently in a supplemental information disclosure statement. The results of experiments with 5D3 showed that its ability to bind intact cells can be reduced or destroyed by various agents that interfere with the cell surface conformation of ABCG2 (an alternate name for BCRP, see results pages 10-16 and discussion pages 17-19). Additionally, in experiments with an antibody generated using an N-terminal intracellular epitope of ABCG2, the BXP-21 antibody, that antibody failed to recognize ABCG2 on a living cell (see page 17).

Applicants also note that antibody BXP-34, described by Scheffer *et al.*, 2000, Cancer Res.; 60:2589-2593 (a copy of which is being submitted concurrently in a supplemental information disclosure statement), was unreactive in Western blots with protein extracts of BCRP. However, BXP-34 was "unable to stain viable unfixed BCRP-positive cells, which showed that the Mab detects an internal epitope of the BCRP protein." (see pages 2591-2592). Thus, neither BXP-21 nor BXP-34 was capable of binding specifically to a native human or murine ABCG2/BCRP conformation expressed on living cells, and these failures highlight the striking success of the inventive antibodies exemplified by 5D3.

Importantly, 5D3 fails to react with ABCG2 on immunoblots, illustrating that 5D3 does not bind to denatured, purified ABCG2 (see page 10). These results illustrate that 5D3 exhibits binding specificity for ABCG2 as it is expressed on the cell surface and show that it fails to recognize denatured ABCG2 (i.e., denatured BCRP).

The Examiner's attempt to combine Mechetner and Ross also fails because there is no suggestion in the prior art to combine these references. Both the motivation to combine the relevant elements and the suggestion of success must be found in the prior art to satisfy the requirements for maintaining an obviousness rejection. *In re The Dow Chemical Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988) ("[b]oth the suggestion and the expectation of success must be founded in the prior art, not in the applicant's disclosure"). The mere mention of elements in different references is not sufficient motivation to combine them to arrive at a claimed invention. *In re Rouffet*, 47 USPQ2d 1453 (Fed. Cir. 1998) ("[T]he examiner must show reasons that the skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would *select the elements from the cited prior art references for combination in the manner claimed.")* (citations omitted, emphasis added).

With regard to the previous arguments and declarations, Applicants note that prior to the inventors' disclosure, no one had successfully reported the production of antibodies that would bind specifically to a native human or murine BCRP conformation expressed on living cells. The prevailing sentiment by those skilled in the art was that such techniques would NOT work for this purpose as the Sarkadi declaration explains (at page 2). Contrary to expectations, the inventors successfully made the desired antibody, as exemplified through four hybridomas: 5D3, 7A3, 1C5 and 8C2. The inventors have created a new class of antibody to a structurally well-characterized antigen. There is nothing contradictory in this outcome.

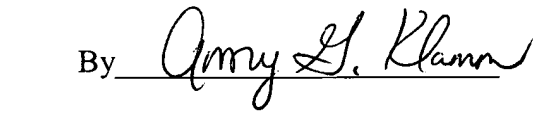
In conclusion, because combining Ross and Mechetner fails to either teach or suggest the claimed antibodies, and since there would be no reasonable expectation of successfully making the claimed antibodies following the combined teaching of the references, assuming that the references suggested such an effort, the rejection under 35 U.S.C. §103(a) fails.

CONCLUSION

Applicants respectfully request entry of the foregoing amendments and remarks in the file history of this application. In light of the foregoing amendments and remarks, the specification and pending claims are in condition for allowance. Allowance of the application is earnestly solicited.

Dated: December 20, 2005

Respectfully submitted,

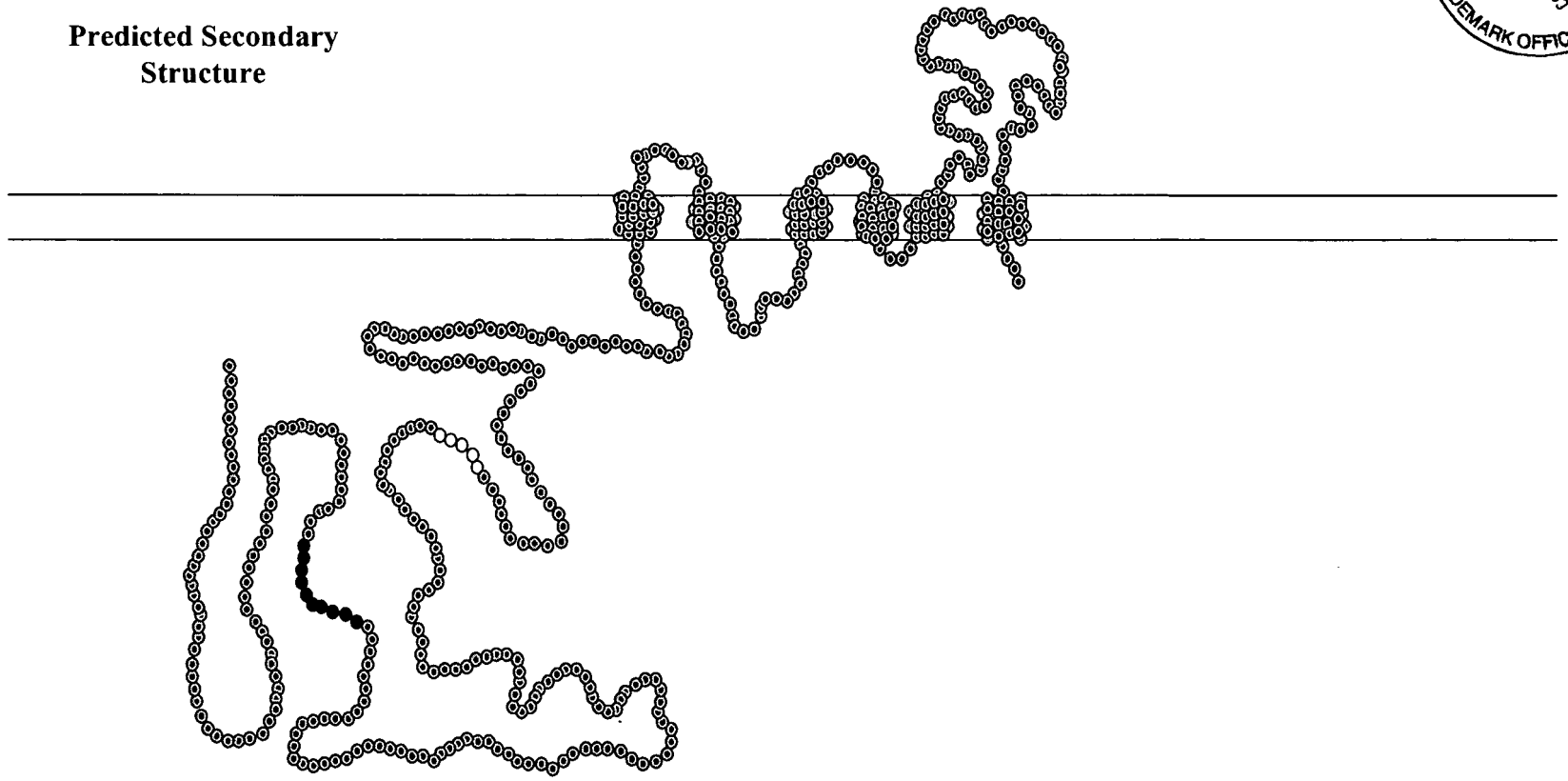
By 

Amy G. Klann, Ph.D.
Registration No.: 48,155
DARBY & DARBY P.C.
P.O. Box 5257
New York, New York 10150-5257
(212) 527-7700
(212) 527-7701 (Fax)
Attorneys/Agents For Applicant

EXHIBIT 1

BCRP

Predicted Secondary Structure



JBC Papers in Press. Published on December 2, 2004 as Manuscript M411338200

FUNCTION-DEPENDENT CONFORMATIONAL CHANGES OF THE ABCG2 MULTIDRUG TRANSPORTER MODIFY ITS INTERACTION WITH A MONOCLONAL ANTIBODY ON THE CELL SURFACE

Csilla Özvegy-Laczka[1,2*], György Várady[3*], Gabriella Köblös[2], Olga Ujhelly[3], Judit Cervenak[3], John D. Schuetz[4], Brian P. Sorrentino[4], Gerrit-Jan Koomen[5], András Váradi[3], Katalin Német[3] and Balázs Sarkadi[1*]

[1]National Medical Center, Institute of Haematology and Immunology, Membrane Research Group, Hungarian Academy of Sciences, 1113 Budapest, Hungary,

[2]Institute of Enzymology, Hungarian Academy of Sciences, 1113 Budapest, Hungary,

[3]National Medical Center, Institute of Haematology and Immunology, Experimental Gene Therapy Unit, 1113 Budapest, Hungary,

[4]Department of Pharmaceutical Sciences and Department of Hematology, St Jude Children's Research Hospital, Memphis, Tennessee, USA,

[5]Laboratory of Organic Chemistry, Institute of Molecular Chemistry, University of Amsterdam, The Netherlands.

Keywords: multidrug transporter, ABCG2, 5D3 monoclonal antibody, conformation-dependent antibody interaction

Running title: Conformation-sensing ABCG2 antibody

+ Authors contributed equally

***Corresponding author:** Balázs Sarkadi, M.D., Ph.D., National Medical Center, Institute of Haematology and Immunology, 1113 Budapest, Diószegi u. 64, Hungary. Phone/FAX: (36)-1-372-4353, e-mail: sarkadi@biomembrane.hu

Abbreviations:

ABCG2: human MXR/BCRP/ABCP multidrug transporter; ABC transporters: ATP-Binding Cassette transporters; AMP-PNP: Adenosine 5'-(β,γ-imido)triphosphate; DFP: diisopropyl-fluorophosphate; FP: flavopiridol; GAM-PE: goat anti mouse phycoerythrin conjugated secondary antibody; mAb: monoclonal antibody; MDR1: human multidrug resistance protein (P-glycoprotein, ABCB1); MRP1: human multidrug resistance protein 1, ABCC1; MX: mitoxantrone; PFA: paraformaldehyde; Sf9 cells: *Spodoptera frugiperda* ovarian cells; V_i: sodium-orthovanadate.

ABSTRACT

The human ABCG2 protein is an important primary active transporter for hydrophobic compounds in several cell types, and its overexpression causes multidrug resistance in tumors. A monoclonal antibody (5D3) recognizes this protein on the cell surface. In ABCG2-expressing cells 5D3 antibody showed a saturable labeling and inhibited ABCG2 transport and ATPase function. However, at low antibody concentrations 5D3 binding to intact cells depended on the actual conformation of the ABCG2 protein. ATP depletion, or the addition of the ABCG2-inhibitor Ko143, significantly increased, while the vanadate-induced arrest of ABCG2 strongly decreased 5D3 binding. The binding of the 5D3 antibody to a non-functional ABCG2 catalytic center mutant (K86M) in intact cells was not affected by the addition of vanadate, while still increased by Ko143. In isolated membrane fragments the ligand modulation of 5D3 binding to ABCG2 could be analyzed in detail. In this case 5D3 binding was maximum in the presence of ATP, ADP or Ko143, while the non-hydrolysable ATP analog, AMP-PNP, and nucleotide trapping by vanadate, decreased antibody binding. In membranes, expressing the ABCG2-K86M mutant, both ATP, ADP and AMP-PNP decreased, while Ko143 increased 5D3 binding. Based on these data we suggest that the 5D3 antibody can be used as a sensitive tool to reveal intramolecular changes, reflecting ATP binding, the formation of a catalytic intermediate, or substrate inhibition within the transport cycle of the ABCG2 protein.

INTRODUCTION

The ABCG2 (MXR/BCRP/ABCP) protein causes multidrug resistance in cancer cells and may have an important function in physiological protection of various tissues against toxic agents. ABCG2 was first cloned from the placenta, where it is most abundantly expressed (1). The overexpression of ABCG2 was observed in certain drug-resistant cell lines and tumors, providing a special multidrug resistant phenotype in these cancer cells (2-5). The ABCG2 protein is a so-called ABC half-transporter, which has only one nucleotide binding (ABC) and one transmembrane domain, and most probably works as a homodimer in the plasma membrane (6-11).

The overexpression of ABCG2 was documented in several human tumors, which indicates its possible importance in the multidrug resistant phenotype of various cancer cells (12-15). The substrate specificity of ABCG2 partially overlaps with the other major multidrug resistance ABC transporters, MDR1 and MRP1, that is the compounds transported by ABCG2 are also large, hydrophobic molecules, including mitoxantrone, topotecan, flavopiridol, methotrexate and Hoechst 33342 (13,16,17).

ABCG2 was found to be physiologically expressed in the liver, small intestine, colon, lung, kidney, adrenal and sweat glands, and in the endothelia of veins and capillaries. The functional characteristics and the tissue distribution of ABCG2 suggest a major role in the tissue protection against xenobiotics (4,13,18). High level expression of the ABCG2 protein and its fluorescent dye extrusion function has been suggested for the identification of bone marrow stem cells (17). Moreover, this so called "side population" of progenitor cells, actively extruding the fluorescent Hoechst 33342 dye, seems to contain pluripotent stem cells in a variety of tissue sources (17,19-21).

The proper detection of the ABCG2 protein would be of major importance in cancer diagnostics, as well as in stem cell research and stem-cell based therapeutic developments. The recent development of a monoclonal antibody, specifically reacting with the human ABCG2 protein on the cell surface (17) has been a major breakthrough in this regard. This antibody was prepared by immunizing mice with intact mouse fibroblasts, expressing the human ABCG2. The antibody, named 5D3, was reported to inhibit the Hoechst 33342 dye transport function of ABCG2 in intact cells (22), and was made commercially available (eBioscience). Similar antibodies have already been prepared against the human MDR1 multidrug transporter (23,24). In the case of MDR1, several of the mAbs reacting with

extracellular epitopes were found to inhibit the transport function of the protein, and the reactivity of one of these antibodies, UIC2 was reported to depend on the conformation of the MDR1 protein (23,25-27).

In the present experiments we have studied the interaction of the anti-ABCG2 monoclonal antibody 5D3 in various cell types expressing the human ABCG2 protein, and examined the effects of ABCG2 protein modulators on this interaction. We have also compared these effects to those of cell fixation and/or permeabilization, and correlated ABCG2 protein detection with another monoclonal antibody, raised against an intracellular epitope of human ABCG2, BXP-21 (28). We also show here that 5D3 binding to ABCG2 in isolated membrane fragments can be analyzed, which allows a detailed investigation of the ligand modulation of antibody binding.

We found that the interaction of 5D3 with ABCG2 was strongly dependent on the modulation of the multidrug transporter protein, thus 5D3 binding to an extracellular ABCG2 epitope was conformation-sensitive. Based on these data, and on previous results for the interaction of human MDR1 protein with conformation-sensitive antibodies, we suggest a model for the transport cycle dependence of 5D3 antibody interaction with the ABCG2 protein. Our data indicate that this conformation-sensitive antibody interaction can be applied for studying the molecular mechanism and the detection of ligand interactions of ABCG2.

EXPERIMENTAL PROCEDURES

Materials

Mitoxantrone, Na-orthovanadate, propidium iodide, AMP, ADP, AMP-PNP and ATP were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes. BXP-21 antibody was obtained from Drs. George Scheffer and Rik Scheper (Department of Pathology Free University, Medical Center, Amsterdam, The Netherlands).

Cell lines and retroviral transduction

Retrovirus producing cells and HEK 293T cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The human PLB985 (in the following PLB) cells were kindly provided by Dr. M. Dinauer (Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN), the MCF-7 parental cells and the MCF-7/MX cells were gifts of Dr. Susan E. Bates (Cancer Therapeutics Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD). PG13 (29) was obtained from the American Type Culture Collection, (Rockville, MD, USA). The construction of the ABCG2 retroviral vectors and cell transduction methods were described in detail in (30). Transduced cells in some cases were selected by stepwise increases in mitoxantrone or flavopiridol concentrations or single-cell cloned for the desired level of protein expression. Sf9 cells expressing the ABCG2 protein or its K86M variant were prepared as described previously (31). In the present study we used the K86M variant introduced into the wild type (R482) ABCG2, by cloning the NotI-SpeI fragment of pAcUW21-L/K86M-R482G (31) into the corresponding site of the pAcUW21-L/R482 vector.

Immunodetection of ABCG2

For immunoblotting washed cells were suspended in the presence of 2 mM DFP in 2 × Laemmli buffer and sonicated for 3 × 5 seconds at 4°C. Sf9 membranes were also suspended in Laemmli buffer. The proteins separated on 7.5 % SDS-polyacrylamide gels were electroblotted onto PVDF membranes, and immuno-detection was performed by using the monoclonal antibody BXP-21 (500 × dilution), and a HRP-conjugated goat anti-mouse IgG (5,000 × dilution, Jackson Immunoresearch). Enhanced chemiluminescence (ECL) technique was applied to detect HRP activity on the blots.

For measuring ABCG2 expression by flow cytometry (Becton Dickinson FACS Calibur) 5D3 primary antibody (purified anti-human ABCG2, clone 5D3, e-Bioscience, Cat. No. 14-8888) or BXP-21 antibody and phycoerythrin-labeled anti-mouse secondary

antibody (GAM-PE, Beckman-Coulter) were used. 5D3 binding in intact cells was examined by suspending the cells in phenol red-free Hank's balanced salt solution with additional pH stabilization by 20 mM phosphate buffer. Aliquots of the suspension, containing 3×10^5 cells were incubated with 500 times diluted 5D3 primary antibody (1 μg/ml), 100 times diluted BXP-21 antibody, or mouse IgG2b (1 μg/ml, as isotype control) in 50 μl buffer for 45 minutes at 37°C (all labeling experiments were carried out in shaker water bath). After washing the cells with Hank's solution, containing 0.5% Bovine Serum Albumin (BSA), the cells were labeled by 200 times diluted goat anti mouse phycoerythrin conjugated secondary antibody (GAM-PE, 3 μg/ml), in 50μl buffer for 30 minutes at 37°C. After washing, the cells were resuspended in Hank's medium and 5D3 binding was determined at 488 nm excitation and 585/42 nm emission (FL2) wavelengths.

When the labeling was carried out with PFA-prefixed cells, the cells were incubated in 200 μl of PBS (phosphate buffered saline) solution containing 1% paraformaldehyde for 10 minutes at 37°C before the above mentioned labeling procedure.

For obtaining PFA-fixed and permeabilized cells, the cells were incubated in 200 μl PBS solution, containing 4% paraformaldehyde and 0.05% Triton-X 100, for 10 minutes at 37°C. The same 0.05% Triton-X 100 was present during all steps of the labeling procedure. When labeling was carried out in the presence of modifying agents (5 μM Ko143, 10 mM Na-orthovanadate, 50 μM flavopiridol or 5 μM mitoxantrone), the cells were preincubated with these agents for 10 minutes at 37°C before labeling, and the agents were present during antibody labeling. When applicable, ATP depletion of the cells was carried out before the labeling procedure by washing the cells twice in sugar-free Hank's medium and 30 minutes incubation at 37°C in Hank's medium containing 50 mM 2-deoxy-D-glucose and 15 mM sodium azide. During cell labeling and washing the media contained the same ATP-depleting agents.

Isolated membrane fragments from Sf9 cells (45 μg) were labeled with 1 μg/ml 5D3 (or mouse IgG2b as isotype control) in 100 μl final volume of assay mix (40 mM MOPS-Tris pH 7.0, 5 mM Na-azide, 50 mM KCl, 2 mM DTT and 500 μM EGTA-Tris pH 7.0) for 30 minutes at 37°C. The membranes were then washed with 500 μl assay mix and pelleted at 10,000 g for 4 minutes. The pellet was suspended in assay mix, containing 1 μg/ml GAM-PE, and incubated at 37°C for 30 minutes. The membranes were then washed and centrifuged (10,000 g for 4 minutes). Finally, the pellet was suspended in 200 μl assay mix

and the fluorescence was detected in a fluorescence plate reader (Fluoroskan II, Labsystems) at 485 nm (excitation)/ 590 nm (emission). When the effects of different agents were investigated the membranes were preincubated in assay mix containing 2 mM Na-orthovanadate, 1 μM Ko143, 10 mM MgAMP, MgADP, MgAMP-PNP, MgATP or 10 mM AMP, ADP, AMP-PNP, ATP + 2 mM EDTA or the combination of these agents (as described in the Figure Legends) for 5 minutes at 37°C prior to the addition of the 5D3 antibody. The relative level of 5D3 binding was calculated as follows: $(F_X-F_{IT})/(F_0-F_{IT})*100$. F_X: fluorescence measured in the presence of 5D3 and the investigated compound, F_{IT}: fluorescence measured in the presence of mouse IgG2b (isotype control), F_0: fluorescence measured in the presence of 5D3 alone.

Cellular mitoxantrone uptake

The drug extrusion function of ABCG2 in intact cells was evaluated by the mitoxantrone (MX) uptake assay of Robey et al. (32) as modified by (30). After 5D3 labeling at 37°C for 30 minutes and washing (as described for immuno-labeling), the cells were suspended in phenol red-free Hank's balanced salt solution containing 5 μM MX or 5 μM MX + 5 μM Ko143 (in some experiments 10 mM Na-orthovanadate, or 50 μM flavopiridol) and incubated at 37°C for 30 minutes. After washing, MX fluorescence was analyzed by flow cytometry (FACSCalibur, Becton Dickinson) at 635 nm excitation and 661/16 nm emission wavelengths (FL4). Dead cells were excluded based on propidium iodide (5 μg/ ml) staining.

Measurement of Hoechst 33342 transport activity

Accumulation of Hoechst dye (Hst) was performed by using intact PLB-ABCG2 (R482), PLB-MDR1 or parental PLB cells (30) in a fluorescence spectrophotometer (Perkin Elmer LS 50B) at 350 nm (excitation)/ 460 nm (emission). The cells (3×10^5) were incubated with or without 12 μg 5D3 antibody in 100 μl final volume of the transport buffer (120 mM NaCl, 5 mM KCl, 400 μM $MgCl_2$, 40 μM $CaCl_2$, 10 mM HEPES, 10 mM $NaHCO_3$, 10 mM glucose and 5 mM Na_2HPO_4) 37°C for 30 minutes. Hoechst transport was then determined on 5D3 labeled or non-labeled cells, as described (33).

ATPase activity measurement

Sf9 membranes containing human ABCG2, MDR1 or ABCG2-K86M were harvested and their membranes were isolated and stored at -80 °C according to (34,35). ATPase activity was measured as described previously, by determining the liberation of inorganic

phosphate from ATP with a colorimetric reaction (11). When the effect of antibody binding was investigated, membranes were preincubated with anti-ABCG2 5D3 monoclonal antibody (eBioscience) or mouse IgG2b (isotype control, SIGMA) in 20 or 160 μg/ mg membrane concentration for 30 minutes at 37°C and then washed twice in ice-cold buffer (40 mM MOPS-Tris pH 7.0, 50 mM KCl, 2 mM dithiotreitol and 0.5 mM EDTA) prior to the ATPase activity measurement. The figures represent the mean values of at least three independent experiments with duplicates.

RESULTS

Antibody detection of ABCG2

For the immuno-detection of the human ABCG2 protein in various cell types we used two monoclonal antibodies. The BXP-21 antibody was generated against an N-terminal intracellular epitope (aa. 271-396 - see (28)), while mAb 5D3 was produced by immunizing mice with intact mouse fibroblasts expressing the human ABCG2 protein (17). As documented earlier, BXP-21 recognizes the ABCG2 protein both in immunoblots and in permeabilized cells (28). In contrast, the 5D3 antibody could be used to recognize human ABCG2 on the surface of intact cells (17), but not on immunoblots (see below).

Fig. 1 shows immunoblot detection of the human ABCG2 protein in the various cells used in the present study, by mAb BXP-21. Panel A shows expression of human, wild-type ABCG2 or the K86M-ABCG2 variant in isolated membranes of Sf9 insect cells (11).

Panel B shows BXP-21 immunoreactions with cell lysates of PLB cells, engineered to express the wild-type ABCG2 or its K86M mutant variant. The expression level of the K86M variant of ABCG2 was about one third of the expression obtained for the wild-type protein (these cells could not be selected by mitoxantrone – see Experimental procedures and (31)).

Fig. 1, Panel C documents the retrovirally evoked expression of human ABCG2 in HEK-293T cells, and Panel D demonstrates the overexpression of ABCG2 in the mitoxantrone-selected MCF-7 cell derivative (MCF-7/MX), as detected by the BXP-21 antibody. It should be noted that, in accordance with previous results, we did not find any immuno-reactivity of the 5D3 antibody with ABCG2 on immunoblots.

Fig. 2 demonstrates the detection of ABCG2 in the parental and the ABCG2-expressing PLB cells, respectively, by flow cytometry and using the BXP-21 and the 5D3 monoclonal antibodies. In these experiments, each antibody was used in a concentration of 0.2 $\mu g/10^6$ cells.

We found that in the parental PLBs the 5D3 antibody showed no immunoreactivity, even if the cells were fixed by PFA, or fixed and permeabilized by PFA+Triton X-100 treatment (Fig. 2, Panel A). When parental PLB cells were labeled with the BXP-21 antibody (Fig. 2, Panel B), there was some background labeling observed, as compared to the isotype control. However, in these parental cells BXP-21 labeling did not increase upon treatment with PFA or PFA+Triton X-100.

As shown in Figure 2, Panel D, in the case of the ABCG2-expressing PLB cells, there was no reaction with the BXP-21 mAb, unless the cells were both fixed and Triton-permeabilized. In this latter case a significant, ABCG2-dependent labeling of the cells by BXP-21 was found. In contrast, the 5D3 antibody showed a well visible immunoreactivity with the native ABCG2-expressing PLBs (Figure 2, Panel C). This reactivity was increased by PFA fixation, while a further permeabilization with Triton X-100 had no effect on 5D3 binding.

It has to be noted that a similar shift in 5D3 reactivity was found upon PFA fixation, and independent of membrane permeabilization, in all ABCG2 expressing cell types studied, including Sf9 insect cells (not shown here). The 5D3 labeling in this latter cell line indicates that the level or even the absence of N-glycosylation does not influence the interaction of 5D3 antibody with ABCG2.

Inhibition of ABCG2 function by the 5D3 antibody

The data presented in Fig. 2 were obtained with relatively low concentrations of the 5D3 antibody (0.2 μg/ 10^6 cells). By increasing the antibody concentration up to 10 μg/10^6 cells, a saturable level of ABCG2 labeling could be achieved, which was not significantly modified by PFA fixation (Figure 3A).

In order to investigate the effect of 5D3 on the ABCG2 function, we preincubated the PLB-ABCG2 cells with the 5D3 antibody (40 μg/10^6 cells) and then measured Hoechst 33342 dye extrusion. As shown in Fig. 3B, at high 5D3 concentrations (40 μg/10^6 cells), a significant ($p = 0.002$), about 65% inhibition of dye transport was observed. In contrast, 5D3 did not inhibit the Hoechst dye transport measured in MDR1-expressing PLBs. In addition, the anti-MDR1 inhibitory monoclonal antibody, UIC2 inhibited Hoechst 33342 extrusion in the MDR1-expressing cells, while did not modify the transport activity in the PLB-ABCG2 cells (not shown).

In order to further explore the ABCG2 inhibitory potential and selectivity of the 5D3 antibody, we have performed direct ABCG2-ATPase measurements in isolated Sf9 cell membranes (Fig. 3C). In these experiments we preincubated the isolated membranes for 30 min at 37°C with two different 5D3 concentrations (20 μg and 160 μg 5D3/ mg membrane protein, respectively) in the absence of ATP, to assure maximum 5D3 labeling of ABCG2 (see below). We found that the application of the lower, 20 μg/mg membrane 5D3 concentration, although at least 20 times greater than that used in the whole-cell experiments, did not significantly affect the ABCG2-ATPase ($p = 0.1$). However, when the

ATPase activity was measured after labeling with 160 μg 5D3/mg membrane protein, a significant ($p = 0.007$), about 30% decrease in the vanadate-sensitive ATPase activity of ABCG2 was observed. No inhibition was seen in the presence of similar concentrations of an isotype control antibody. There was no effect of 5D3 antibody on the ATPase activity of MDR1 or ABCG2-K86M membranes. All these data indicate that the 5D3 antibody, when applied in high concentrations, specifically inhibits the transport and ATPase function of the ABCG2 protein.

Effects of ABCG2 inhibitors on 5D3 reactivity and mitoxantrone transport by ABCG2 in intact cells

In the following experiments we have studied the effects of a specific ABCG2 inhibitor, Ko143 (36) and the general ABC transporter inhibitor, Na-orthovanadate (V_i) on the binding of 5D3 antibody in intact cells by flow cytometry. The 5D3 labeling conditions were as described for Fig. 2, that is relatively low antibody concentrations were applied. In the same cells we have also measured mitoxantrone (MX) accumulation, by using a different fluorescence detection channel (see Experimental procedures).

As shown in Fig. 4, Panel A, in the parental PLB cells 5D3 reactivity was negligible, and unchanged by the addition of Ko143 or Na-orthovanadate (V_i). MX accumulation in the same cells reached a high level and was unaffected by the presence of Ko143 or V_i (Fig. 4, Panel B).

In the ABCG2-expressing PLBs we found a low, but measurable 5D3 reactivity (Fig. 4. Panel C), which was greatly increased by Ko143, while slightly reduced by the addition of Na-orthovanadate. In the parallel MX uptake experiments (Panel D), in the ABCG2-expressing PLBs MX accumulation was reduced, as compared to that found in the parental cells. ABCG2 inhibition by both Ko143 and V_i significantly increased intracellular MX level, similar to that seen in cells not expressing ABCG2. Cell labeling with 5D3 at these low antibody concentrations did not cause any change in MX uptake.

According to these results, both Ko143 and V_i blocked the ABCG2 transporter function, but Ko143 increased, while V_i rather decreased 5D3 binding on the cell surface. On the other hand, 5D3 labeling at this lower antibody concentrations did not inhibit MX transport activity of ABCG2 (see below).

When we analyzed 5D3 binding and MX uptake in other ABCG2 expressing mammalian cell types, we found a similar modulation of 5D3 binding and MX transport by these inhibitors. The data presented in Fig. 4, Panel G, document that in ABCG2-

transduced HEK-293T cells 5D3 binding was decreased by V_i treatment and increased by Ko143. As shown in Panel H, MX transport in these cells was inhibited by both inhibitors (interestingly, vanadate preincubation could not block MX extrusion in all HEK cells, a variable population of transporting cells was still observed in these experiments). Parental HEK cells did not show a significant ABCG2 expression or MX transport activity (Panels E and F).

We obtained essentially similar data in the MCF-7/MX cells and the PLBs expressing the gain-of function R482G mutant of ABCG2 (not shown). As a summary, the addition of Ko143 and V_i treatment blocked ABCG2 function in all these cell types, and Ko143 significantly increased, while Na-orthovanadate decreased 5D3 binding to the ABCG2 protein.

Effect of ATP depletion and transported substrates on 5D3 reactivity and mitoxantrone transport by ABCG2 in intact cells

In the following experiments we have studied the effect of ATP depletion and various transported substrates on 5D3 binding and MX extrusion by ABCG2 in intact PLB cells. For achieving an efficient ATP depletion of the ABCG2-expressing PLBs, we used a 30 min pretreatment at 37°C, with a combination of Na-azide and 2-deoxy-D-glucose (see Experimental procedures). As documented earlier in many hematopoietic cell lines, this treatment reduces the ATP level below 5% of the original levels and results in the accumulation of both ADP and AMP in the cells.

As shown in Fig. 5, this ATP depletion strongly inhibited the ABCG2 transport function, that is eliminated the ABCG2-dependent MX extrusion in these cells (Panel B). Interestingly, ATP depletion significantly increased 5D3 binding, thus transforming the ABCG2 protein in a conformation optimal for 5D3 labeling (Panel A).

We have examined the effects of various agents on 5D3 binding, which were demonstrated transported substrates of the ABCG2 protein. The co-incubation of the ABCG2 cells with mitoxantrone (2-5 μM) did not influence 5D3 labeling (see Figure 5 Panel C). We also found no appreciable effect on 5D3 binding by the addition of other substrates, prazosin (10-50 μM), or ZD1839 (0.1-1 μM) (not shown) (5,32,33). Flavopiridol (FP), another transported substrate of ABCG2 (37) in low (1-5 μM) concentrations had no effect on 5D3 antibody labeling, while in concentrations above 50 μM this agent significantly increased 5D3 labeling and interfered with MX extrusion (Figure 5 Panels B and C). This is in line with the ABCG2-ATPase measurements, where

high flavopiridol concentrations were inhibitory, thus could act similarly to Ko143 (data not shown in detail).

Effects of substrates, inhibitors and ATP depletion on 5D3 reactivity in the mutant, non-functional K86M-ABCG2, expressed in intact cells

In the next set of experiments we studied intact mammalian cells expressing a non-functional mutant (K86M) variant of ABCG2. This mutation in the highly conserved Walker A motif does not affect ATP binding by ABCG2, but impairs its drug transport and ATPase activity, as well as the formation of a vanadate-induced trapped nucleotide (31). As shown in Fig. 6, Panels A and B, this K86M-ABCG2 had no MX extrusion function, but showed a well measurable 5D3 binding on the cell surface.

In these studies we found that the 5D3 binding of the K86M mutant ABCG2 was significantly increased by PFA fixation, ATP-depletion or Ko143 treatment. Still, the relative increase in 5D3 binding due to these effects was much smaller than in the case of the wt ABCG2, and 5D3 binding was unaffected by pretreatment with Na-orthovanadate (Fig. 6. Panel A). Thus the non-functional K86M variant of ABCG2 showed a relatively high 5D3 binding in its native state, but in the case of ATP-removal and Ko143 treatment similar conformational changes were detected by 5D3 in this mutant variant as in the wild-type protein. The lack of the formation of a transition-state intermediate in the K86M-ABCG2 correlated with the absence of an effect of Na-orthovanadate.

Effects of nucleotides and transport inhibitors on 5D3 reactivity of ABCG2 in isolated membrane fragments

In the following experiments we examined the effects of various nucleotides and transport inhibitors on 5D3 binding by human ABCG2 and its mutant (K86M) variant in isolated insect cell membrane fragments. In these membrane preparations ABCG2 expression reaches a high level (up to 5% of the membrane proteins), in a fully active form, as reflected by the ABCG2-ATPase activity (11,31). A large fraction of the isolated membrane fragments are accessible both from the cytoplasmic and the external cell surface, as tested by the trypsin sensitivity of open fragments (38) and simultaneous staining of the membrane fragments with two antibodies (pAb 405 and mAb 5D3), that recognize an intracellular (5), and an extracellular epitope of ABCG2, respectively (not shown here). Therefore this assay system allows a direct estimation of the effects of cytoplasmic ligands on the cell surface interaction of ABCG2 with the 5D3 antibody.

As shown in Fig. 7, Panel A, 5D3 binding to isolated Sf9 cell membranes, containing the human ABCG2 protein, reached a high level, significantly exceeding that seen in the control, MDR1-containing membranes, or the labeling obtained with an isotype control antibody.

Fig 7 B and C document the effects of various ligands on 5D3 binding to wild-type (Panel B) or K86M (Panel C) ABCG2 in isolated membranes. In the case of the wild-type ABCG2 (Panel B), the addition of MgAMP, MgADP, or MgATP did not significantly modulate the level of 5D3 labeling, while MgAMP-PNP, a non-hydrolysable ATP analog greatly reduced 5D3 binding. The addition of Na-orthovanadate was ineffective in the presence of MgAMP, while produced a major decrease in 5D3 binding together with MgATP. When the cells were preincubated with the transport inhibitor Ko143, either in the presence of MgATP or MgAMP-PNP, a maximum level of 5D3 binding to ABCG2 was observed. Ko143 preincubation produced a maximum 5D3 binding even in the presence of MgATP+vanadate. An interesting finding was in these experiments, that if Ko143 was added after a preincubation with MgAMP-PNP, the reduction in 5D3 binding by this nucleotide could not be reversed by Ko143 (data not shown).

These data indicate that in the case of a functional ABCG2, 5D3 labeling has a relatively high level either in a nucleotide-free, or in a nucleotide-liganded, flexible state of the transporter. However, when the transport cycle is blocked by a non-hydrolysable ATP analog, or by the inhibition of ATP hydrolysis by Na-orthovanadate, a strong reduction in

5D3 binding occurs. Arresting the ABCG2 transport cycle by Ko143, however, produces a high 5D3 binding, and this effect is not reversed the nucleotides and/or vanadate. Still, a low 5D3 binding conformation first fixed by MgAMP-PNP, cannot be changed to a high binding form by a later addition of Ko143.

Fig. 7, Panel C shows 5D3 binding in isolated membranes containing the K86M, non-functional mutant ABCG2 protein. In this case MgAMP had no effect, while both MgATP, MgADP and MgAMP-PNP significantly reduced 5D3 labeling. Na orthovanadate did not modify 5D3 binding, as compared to that seen with the respective nucleotides (MgAMP or MgATP). The addition of Ko143, again even in the presence of MgATP, MgADP, or MgAMP-PNP, produced maximum 5D3 binding.

These data can be interpreted to mean, that while MgAMP does not show binding to the protein, both MgATP, MgADP and MgAMP-PNP are bound to K86M-ABCG2 and, in the absence of a full catalytic cycle, they fix the transporter in a nucleotide-bound, reduced 5D3 binding state. This fixation does not require the presence of vanadate. These findings are in agreement with the unchanged ATP binding, but the lack of vanadate-dependent nucleotide trapping in the case of this mutant protein (31). Interestingly, Ko143 can still stabilize the K86M-ABCG2 variant in a high 5D3 binding state.

In experiments not documented here in detail, we have performed 5D3 binding to ABCG2 in isolated Sf9 membranes at 4°C, in order to investigate labeling at non-hydrolytic conditions. We found that 5D3 binding at 4°C was somewhat reduced (75 ± 1.4% of that measured at 37°C), and the addition of nucleotides or inhibitors (Ko143 or V_i) did not cause a measurable change in 5D3 binding.

We have also investigated 5D3 binding to ABCG2 in isolated Sf9 membranes upon the addition of AMP, ADP, AMP-PNP and ATP, but in the absence of Mg^{2+} ions (that is in the presence of excess EDTA), at 37°C. Interestingly, we found that in the absence of Mg^{2+}, both ADP, AMP-PNP and ATP (but not AMP) significantly decreased 5D3 binding to the ABCG2 protein. These effects were similar both in the wild-type ABCG2 and the K86M mutant variant (not documented in detail). These data indicate that the binding of ADP, ATP or AMP-PNP to ABCG2 (causing low 5D3 reactivity) occurs even in the absence of Mg^{2+}, but no further steps of the catalytic cycle are performed.

DISCUSSION

In the present experiments we have studied the interaction of the 5D3 monoclonal antibody, prepared against a cell surface epitope of human ABCG2, with this multidrug transporter both in intact cells and in isolated membranes. We found that in intact cells 5D3 recognition of the ABCG2 protein occurred at an external epitope. The specific antibody binding was significantly increased by fixation of the intact cells by paraformaldehyde (PFA), but this interaction did not require membrane permeabilization (Figure 2 Panel C). In contrast, the interaction of BXP-21 (an antibody raised against an intracellular epitope) with ABCG2 entirely depended on permeabilization of the cell membranes, making the intracellular epitopes accessible for this antibody (Figure 2 Panel D).

In accordance with data in the literature regarding 5D3 effect on ABCG2-induced drug resistance (22), we found that the 5D3 antibody significantly inhibited both the dye transport and the ATPase activity of the ABCG2 protein (Figure 3, Panels B and C). Still, the inhibition of the transport or ATPase activity of ABCG2 found here was incomplete even at very high 5D3 concentrations (see Figures 3B and 3C). This finding is most probably due to the steric and mechanical constrains in such antibody-transporter interactions. A similarly selective, but only partial functional inhibition has been reported for several anti-MDR1 antibodies, e.g. MRK16 or UIC2, reacting with cell surface epitopes of the MDR1 multidrug transporter (23,24).

In this study we found that at low 5D3 concentrations the actual conformation of the ABCG2 protein significantly modified 5D3 binding to the extracellular epitope. In intact cells ABCG2 interaction with 5D3 was greatly increased by the inhibition of ABCG2 function with a specific, high affinity inhibitor, Ko143 (see Figure 4, Panels C and G), or by cellular ATP depletion. (Fig. 5, Panel A). Similarly, an increase in 5D3 reactivity was observed in the presence of high, inhibitory concentrations of a drug substrate of ABCG2, flavopiridol (Figure 5C) (37).

In contrast, a reduction in 5D3 binding was observed when the cells were preincubated with Na-orthovanadate, a transition-state inhibitor of ABC transporters, including ABCG2 (31,39-41). In this case, within the nucleotide binding domain of the protein, vanadate anions replace phosphate after ATP hydrolysis, and the transport cycle of ABCG2 is arrested in a transition-state. This can be experimentally followed by measuring the

vanadate-dependent trapping of MgADP within the protein, which becomes incapable for further ATPase or transport activity (39-41). In the present experiments the arrest of the ABCG2 transport cycle by Ko143, by the removal of the energy donor substrate, ATP, as well as by Na-orthovanadate was documented by the lack of active mitoxantrone (MX) extrusion in the same cells (Figure 4, Panels D and H, Figure 5, Panel B). In these experiments the addition of low concentrations of transported substrates did not significantly modify cell surface 5D3 binding to ABCG2 (Figure 5, Panel C).

According to these data, 5D3 interaction with ABCG2 in intact cells depends on the actual conformation within the transport cycle of this multidrug resistance protein. 5D3 binding is relatively low in the case of the actively functioning protein or in its stabilized transition state. In contrast, 5D3 binding is greatly increased when ABCG2 conformation is stabilized in other specific conformations (by Ko143 or ATP depletion). In unpublished experiments we found that Ko143 inhibition of ABCG2 was reversible by repeated washings. Also, ABCG2-ATPase inhibition achieved by low (10 nM) Ko143 concentration could be removed by the addition of increasing concentrations of transported substrates, e.g. prazosin. These results indicate that Ko143 probably inhibits ABCG2 by interacting with its substrate binding site.

When examining the binding of the 5D3 antibody in intact cells to a non-functional ABCG2 catalytic center mutant (K86M-ABCG2), we found that 5D3 binding to this mutant protein was also efficient. In the case of this mutant ABCG2, 5D3 binding was not affected by the addition of transported substrates or vanadate, while it was increased by ATP-depletion or by the addition of Ko143 (Figure 6). These data are in line with the impaired catalytic cycle and transition state forming ability of this mutant, with unchanged ATP binding (31), and probably with conserved drug/inhibitor binding properties.

In order to further explore the mechanistic details of the ABCG2 catalytic cycle, we have performed a detailed analysis of 5D3 binding to ABCG2 in isolated membrane fragments, accessible from both sides of the membrane (see Fig. 7, Panel B). It is important to note that the experiments carried out with isolated membranes exclude the possibility that the changes in 5D3-ABCG2 interactions might be due to variable cell surface expression of the multidrug resistance protein in intact cells. They also allow to study the interaction of non cell-permeating ligands with cytoplasmic domains of the transporter.

In these experiments we observed that the non-hydrolysable ATP analog, AMP-PNP, strongly reduced 5D3 binding to ABCG2. MgAMP, MgADP, or MgATP had no major effect, but MgATP+Na-orthovanadate induced a major decrease in 5D3 binding. Preincubation with the inhibitor molecule, Ko143 maximized 5D3 binding under all conditions.

In the K86M-ABCG2 variant, the addition of MgATP, MgADP and MgAMP-PNP, all caused a major reduction of 5D3 binding, which was not further modulated by Na-orthovanadate. These results coincide with the conserved ATP binding, but impaired catalytic intermediate formation by this mutant protein. In the case of this non-functional mutant we still found an increased 5D3 binding upon preincubation with Ko143, even if MgATP, MgADP or MgAMP-PNP were added thereafter to the media (see Fig. 7, Panel C). These results suggest a preserved substrate/inhibitor binding site in this mutant protein.

Interestingly, in Sf9 membranes containing either wild-type ABCG2 or its K86M mutant, in the absence of Mg^{2+} (that is in the presence of EDTA), both ATP, ADP and AMP-PNP caused a decrease in 5D3 binding (not shown in detail). These data may indicate that at these high nucleotide concentrations (10 mM), ABCG2 binds nucleotides even in the absence of Mg^{2+}, although further ATP hydrolysis is absent.

When trying to investigate the possible effects of transported substrates (e.g. prazosin, flavopiridol, or mitoxantrone) on 5D3 binding by ABCG2 in isolated Sf9 cell membrane fragments, we could not detect any major changes evoked by relevant substrate concentrations. This is similar to the lack on ATPase stimulation by substrates in this system, and most probably due to the presence of endogenous substrates of ABCG2 in the Sf9 membranes (31).

These data collectively indicate that the binding of the 5D3 monoclonal antibody closely reflects the changes in the drug- and ATP binding, as well as the catalytic state of the ABCG2 transporter. This is most probably due to the variable appearance of a conformational epitope within the ABCG2 protein on the cell surface. This study is the first demonstration of such a conformation-sensitivity of an antibody binding to the ABCG2 protein, although a conformation dependent binding of some extracellular antibodies, e.g. MRK16 or UIC2, to another multidrug transporter, the MDR1 protein, has already been documented (23,24). The determination of the actual epitope structure involved in 5D3 binding should require a detailed molecular mapping of potentially cell-surface domains of ABCG2.

As a summary, the various steps within the catalytic cycle of the ABCG2 multidrug resistance transporter could be visualized through changes in 5D3 binding. A low level 5D3 binding was observed when the non-hydrolysable ATP analog, MgAMP-PNP, or the addition of ATP or ADP without Mg^{2+} ions stabilized the protein in a pre-hydrolytic state (42,43). The formation of a catalytic intermediate, reflected by nucleotide trapping in the presence of vanadate anions (40,41), also coincided with a low 5D3 reactivity of ABCG2. In contrast, transport inhibition by Ko143 or by high concentrations of flavopiridol, as well as by ATP depletion, stabilized the protein in a conformation with high 5D3 binding capacity.

Based on these data we suggest that the 5D3 reactive form of ABCG2 is a stabilized "substrate off-site" conformation of the transporter. It has to be noted that the ABCG2 protein is an ABC half-transporter, and its function requires homo-dimerization (6-8,11). Conformational changes detected through a complex extracellular epitope of a membrane protein can be due to a function-dependent rearrangement of the transmembrane helices, triggering the movements of the extracellular loops, or to the surface-exposure of membrane-embedded short segments (for MDR1 see (44)). ABCG2 acts as a homodimer, and one additional possible explanation for the conformational changes described in the present study is the function-dependent re-orientation of the monomers within the dimer, or facilitation of the dimer formation. However, further experiments are needed to elucidate the dependence of 5D3 binding on the molecular interactions between the dimerizing ABCG2 molecules.

Based on this study we suggest that the 5D3 antibody can be used to reveal major intramolecular changes in the ABCG2 protein during its catalytic/transport cycle. Examining 5D3 binding to various mutant, polymorphic, or stabilized forms of ABCG2 may further help structure-function relationship studies. Moreover, based on the present data, optimum conditions can be selected for the investigation of ABCG2 expression and function by 5D3 binding in intact cell preparations, thus employing this antibody for a sensitive clinical laboratory detection of ABCG2 expression and function.

ACKNOWLEDGMENTS

The technical help by Zsuzsanna Andrási, Judit Kis, Mónika Bátkai, Margit Bakki and Katalin Kelemen is gratefully acknowledged. This work has been supported by research grants from OTKA and OM, Hungary (T-29921, T-35126, T-31952 and NKFP 1/024/2001 and OM-2568/1999). Csilla Özvegy-Laczka is a grantee of the Postdoctoral Fellowship (D45957) of OTKA, Hungary and the János Bolyai Scholarship of the Hungarian Academy of Sciences. Balázs Sarkadi is a recipient of a Howard Hughes International Scholarship.

REFERENCES

1. Allikmets, R., Schriml, L. M., Hutchinson, A., Romano-Spica, V., and Dean, M. (1998) *Cancer Res* **58**, 5337-5339
2. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. (1998) *Proc Natl Acad Sci U S A* **95**, 15665-15670
3. Allen, J. D., Brinkhuis, R. F., Wijnholds, J., and Schinkel, A. H. (1999) *Cancer Res* **59**, 4237-4241
4. Maliepaard, M., van Gastelen, M. A., de Jong, L. A., Pluim, D., van Waardenburg, R. C., Ruevekamp-Helmers, M. C., Floot, B. G., and Schellens, J. H. (1999) *Cancer Res* **59**, 4559-4563
5. Litman, T., Brangi, M., Hudson, E., Fetsch, P., Abati, A., Ross, D. D., Miyake, K., Resau, J. H., and Bates, S. E. (2000) *J Cell Sci* **113 (Pt 11)**, 2011-2021
6. Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T., and Bates, S. E. (1999) *Cancer Res* **59**, 8-13
7. Knutsen, T., Rao, V. K., Ried, T., Mickley, L., Schneider, E., Miyake, K., Ghadimi, B. M., Padilla-Nash, H., Pack, S., Greenberger, L., Cowan, K., Dean, M., Fojo, T., and Bates, S. (2000) *Genes Chromosomes Cancer* **27**, 110-116
8. Kage, K., Tsukahara, S., Sugiyama, T., Asada, S., Ishikawa, E., Tsuruo, T., and Sugimoto, Y. (2002) *Int J Cancer* **97**, 626-630
9. Rocchi, E., Khodjakov, A., Volk, E. L., Yang, C. H., Litman, T., Bates, S. E., and Schneider, E. (2000) *Biochem Biophys Res Commun* **271**, 42-46
10. Scheffer, G. L., Maliepaard, M., Pijnenborg, A. C., van Gastelen, M. A., de Jong, M. C., Schroeijers, A. B., van der Kolk, D. M., Allen, J. D., Ross, D. D., van der Valk, P., Dalton, W. S., Schellens, J. H., and Scheper, R. J. (2000) *Cancer Res* **60**, 2589-2593
11. Ozvegy, C., Litman, T., Szakacs, G., Nagy, Z., Bates, S., Varadi, A., and Sarkadi, B. (2001) *Biochem Biophys Res Commun* **285**, 111-117
12. Ross, D. D., Karp, J. E., Chen, T. T., and Doyle, L. A. (2000) *Blood* **96**, 365-368
13. Litman, T., Druley, T. E., Stein, W. D., and Bates, S. E. (2001) *Cell Mol Life Sci* **58**, 931-959

14. Bates, S. E., Robey, R., Miyake, K., Rao, K., Ross, D. D., and Litman, T. (2001) *J Bioenerg Biomembr* **33**, 503-511
15. Diestra, J. E., Scheffer, G. L., Catala, I., Maliepaard, M., Schellens, J. H., Scheper, R. J., Germa-Lluch, J. R., and Izquierdo, M. A. (2002) *J Pathol* **198**, 213-219
16. Volk, E. L., Farley, K. M., Wu, Y., Li, F., Robey, R. W., and Schneider, E. (2002) *Cancer Res* **62**, 5035-5040
17. Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., and Sorrentino, B. P. (2001) *Nat Med* **7**, 1028-1034
18. Cooray, H. C., Blackmore, C. G., Maskell, L., and Barrand, M. A. (2002) *Neuroreport* **13**, 2059-2063
19. Scharenberg, C. W., Harkey, M. A., and Torok-Storb, B. (2002) *Blood* **99**, 507-512
20. Kim, M., Turnquist, H., Jackson, J., Sgagias, M., Yan, Y., Gong, M., Dean, M., Sharp, J. G., and Cowan, K. (2002) *Clin Cancer Res* **8**, 22-28
21. Lechner, A., Leech, C. A., Abraham, E. J., Nolan, A. L., and Habener, J. F. (2002) *Biochem Biophys Res Commun* **293**, 670-674
22. Abbott, B. L., Colapietro, A. M., Barnes, Y., Marini, F., Andreeff, M., and Sorrentino, B. P. (2002) *Blood* **100**, 4594-4601
23. Mechetner, E. B., and Roninson, I. B. (1992) *Proc Natl Acad Sci U S A* **89**, 5824-5828
24. Hamada, H., and Tsuruo, T. (1986) *Proc Natl Acad Sci U S A* **83**, 7785-7789
25. Mechetner, E. B., Schott, B., Morse, B. S., Stein, W. D., Druley, T., Davis, K. A., Tsuruo, T., and Roninson, I. B. (1997) *Proc Natl Acad Sci U S A* **94**, 12908-12913
26. Goda, K., Nagy, H., Bene, L., Balazs, M., Arceci, R., Mechetner, E., and Szabo, G. (2000) *Cancer Detect Prev* **24**, 415-421
27. Nagy, H., Goda, K., Arceci, R., Cianfriglia, M., Mechetner, E., and Szabo, G., Jr. (2001) *Eur J Biochem* **268**, 2416-2420
28. Maliepaard, M., Scheffer, G. L., Faneyte, I. F., van Gastelen, M. A., Pijnenborg, A. C., Schinkel, A. H., van De Vijver, M. J., Scheper, R. J., and Schellens, J. H. (2001) *Cancer Res* **61**, 3458-3464
29. Miller, A. D., Garcia, J. V., von Suhr, N., Lynch, C. M., Wilson, C., and Eiden, M. V. (1991) *J Virol* **65**, 2220-2224

30. Ujhelly, O., Ozvegy, C., Varady, G., Cervenak, J., Homolya, L., Grez, M., Scheffer, G., Roos, D., Bates, S. E., Varadi, A., Sarkadi, B., and Nemet, K. (2003) *Hum Gene Ther* **14**, 403-412
31. Ozvegy, C., Varadi, A., and Sarkadi, B. (2002) *J Biol Chem* **277**, 47980-47990
32. Robey, R. W., Honjo, Y., van de Laar, A., Miyake, K., Regis, J. T., Litman, T., and Bates, S. E. (2001) *Biochim Biophys Acta* **1512**, 171-182
33. Ozvegy-Laczka, C., Hegedus, T., Varady, G., Ujhelly, O., Schuetz, J. D., Varadi, A., Keri, G., Orfi, L., Nemet, K., and Sarkadi, B. (2004) *Mol Pharmacol* **65**, 1485-1495
34. Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., and Scarborough, G. A. (1992) *J Biol Chem* **267**, 4854-4858.
35. Sarkadi, B., Bauzon, D., Huckle, W. R., Earp, H. S., Berry, A., Suchindran, H., Price, E. M., Olson, J. C., Boucher, R. C., and Scarborough, G. A. (1992) *J Biol Chem* **267**, 2087-2095
36. Allen, J. D., van Loevezijn, A., Lakhai, J. M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J. H., Koomen, G. J., and Schinkel, A. H. (2002) *Mol Cancer Ther* **1**, 417-425
37. Robey, R. W., Medina-Perez, W. Y., Nishiyama, K., Lahusen, T., Miyake, K., Litman, T., Senderowicz, A. M., Ross, D. D., and Bates, S. E. (2001) *Clin Cancer Res* **7**, 145-152
38. Sinko, E., Ilias, A., Ujhelly, O., Homolya, L., Scheffer, G. L., Bergen, A. A., Sarkadi, B., and Varadi, A. (2003) *Biochem Biophys Res Commun* **308**, 263-269
39. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. (1995) *J Biol Chem* **270**, 19383-19390
40. Taguchi, Y., Yoshida, A., Takada, Y., Komano, T., and Ueda, K. (1997) *FEBS Lett* **401**, 11-14
41. Szabo, K., Welker, E., Bakos, Muller, M., Roninson, I., Varadi, A., and Sarkadi, B. (1998) *J Biol Chem* **273**, 10132-10138
42. Hou, Y. X., Cui, L., Riordan, J. R., and Chang, X. B. (2002) *J Biol Chem* **277**, 5110-5119
43. Sauna, Z. E., and Ambudkar, S. V. (2001) *J Biol Chem* **276**, 11653-11661
44. Rosenberg, M. F., Kamis, A. B., Callaghan, R., Higgins, C. F., and Ford, R. C. (2003) *J Biol Chem* **278**, 8294-8299

LEGENDS FOR THE FIGURES

Figure 1. Immunoblot detection of human ABCG2 by monoclonal antibody, BXP-21.

Sf9 membranes containing wtABCG2, ABCG2-K86M or β-galactosidase (Panel A) and cell lysates from PLB (Panel B), HEK 293 (Panel C) and MCF-7/MX (Panel D) cells expressing wtABCG2 (or ABCG2-K86M) or parental cells (ctr.) were subjected to Laemmli gel electrophoresis and electroblotting. The amount of protein samples loaded on the gel were 2 μg for Sf9 membranes, HEK and MCF-7 cells, and 40 μg for PLB cells lysates. ABCG2 was detected by the BXP-21 monoclonal antibody. Experiments were performed three times, the Figure shows the result of one representative experiment.

Figure 2. Flow cytometry detection of ABCG2 in the parental PLB (Panel A and B), and the ABCG2-expressing PLB cells (Panel C and D), by the 5D3 and the BXP-21 monoclonal antibodies. Effect of fixation by PFA and permeabilization by PFA+Triton X-100.

PLB cells were treated with 1 % PFA (dashed line) or 4 % PFA + 0.05% Triton-X 100 (heavy solid line) prior to 5D3 (left panels) or BXP-21 (right panels) labeling. Non-treated PLB cells (native, solid line) were also labeled with one of the monoclonal antibodies or isotype control (IT, dotted line). Fluorescence of phycoerythrin conjugated secondary antibody was analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Figure shows the result of one representative experiment.

Figure 3. Effect of 5D3 antibody concentration on the labeling (Panel A) or function of ABCG2 (Panels B and C).

Panel A: Labeling of PLB cells by 5D3.

PLB parental (left) or wtABCG2 expressing (right) cells were incubated with different concentrations of the 5D3 antibody: 0.2 μg 5D3/ 10^6 cells (L 5D3, solid line) or 10 μg 5D3/ 10^6 cells (H 5D3, dotted line) or isotype control (IT, dotted line). Cells fixed with 1 % PFA were also labeled with 0.2 μg (L 5D3+PFA, heavy solid line) or 10 μg (H 5D3+PFA, dashed line) / 10^6 cells 5D3 concentrations. Fluorescence of phycoerythrin conjugated secondary antibody was analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Panel B: Inhibition of the Hoechst 33342 dye transport by the 5D3 antibody in PLB cells.

3 x 10^5 PLB cells expressing wtABCG2 or MDR1 and control cells were incubated with (5D3, black columns) or without (control, white columns) 12 μg 5D3 and then Hoechst transport activity was measured in a fluorescence spectrophotometer (Perkin Elmer LS 50B) at 350 nm (excitation)/ 460 nm (emission). Hoechst transport was determined as described in Experimental procedures.

Panel C: Inhibition of the ABCG2-ATPase activity in isolated Sf9 cell membranes by the 5D3 antibody.

Sf9 membranes containing wtABCG2, ABCG2-K86M or MDR1 were incubated with 20 (low 5D3, hatched columns) or 160 μg (high 5D3, black columns) / mg membrane concentration of 5D3 antibody. ATPase activity was determined in 5D3 labeled or non-labeled (control, white columns) membranes by measuring vanadate sensitive inorganic phosphate liberation by colorimetric detection of inorganic phosphate liberation. Data points represent the mean ± standard deviation (S.D.) values of at least four measurements.

Figure 4. Flow cytometry detection of 5D3 mAb binding and mitoxantrone (MX) extrusion by ABCG2 in intact cells. Effects of Ko143 and Na-orthovanadate.

Panels A and B show parental PLB cells, Panels C and D represent ABCG2-expressing PLBs. Panels E and F show parental HEK293 cells, G and H show HEK293 cells expressing ABCG2.

Cells were incubated with 0.2 μg 5D3 antibody / 10^6 cells without (5D3, solid line) or with the addition of 5 μM Ko143 (dashed line) or 2 mM Na-orthovanadate (Vi, heavy solid line). IT means isotype control (dotted line). Mitoxantrone (MX) accumulation was measured on 5D3-labeled cells in the absence (5D3, solid line) or presence of 5 μM Ko143 (dashed line) or 2 mM Na-orthovanadate (heavy solid line). Experiments were performed three-times. Figure shows the result of one representative experiment.

Figure 5. Effects of ATP depletion and transported substrates on 5D3 binding (Panel A and C) and MX extrusion (Panel B) in ABCG2-expressing intact PLB cells.

PLB-ABCG2 cells were incubated in medium containing 50 mM 2-deoxy-D-glucose and 15 mM sodium azide (ATP depl., heavy solid line), 5 μM Ko143 (dashed line), 50 μM flavopiridol (FP, heavy solid line on Panel C or dotted line on Panel B) or 5 μM mitoxantrone (MX, dashed line) during the 5D3 labeling (Panels A and C) or MX

accumulation assay (Panel B). IT means isotype control. Experiments were performed three-times. Figure shows the result of one representative experiment.

Figure 6. Flow cytometry detection of the K86M-ABCG2 protein. 5D3 mAb binding (Panel A) and MX extrusion (Panel B) in K86M mutant ABCG2-expressing intact PLB cells. Effects of Ko143, ATP-depletion and Na-orthovanadate.

Cells were incubated with 0.2 μg 5D3 antibody / 10^6 cells without (5D3, solid line) or with the addition of 5 μM Ko143 (dashed line), 2 mM Na-orthovanadate (Vi, dashed line) or 50 mM 2-deoxy-D-glucose and 15 mM sodium azide (ATP depl., heavy solid line).

Figure 7. Detection of 5D3 mAb binding to ABCG2 in isolated Sf9 membrane fragments. Effects of nucleotides, Ko143, and Na-orthovanadate.

Panel A: Comparison of 5D3 and isotype control (IT) antibody binding to isolated Sf9 cell membranes. Isolated membrane fragments (45 μg) from Sf9 cells containing wtABCG2, ABCG2-K86M or MDR1 were labeled with 1 μg/ml 5D3 (black columns) or 1 μg/ml mouse IgG2b as isotype control (white columns). Fluorescence was detected in a fluorescence plate reader (Fluoroskan II, Labsystems) at 485 nm (excitation)/ 590 nm (emission).

Panel B: 5D3 binding to membranes containing wild-type ABCG2,

Panel C: 5D3 binding to membranes containing K86M mutant ABCG2.

Sf9 membranes containing wild-type ABCG2 (Panel B) or K86M mutant ABCG2 (Panels C) were incubated with 5D3 antibody in the presence of 10 mM MgAMP, MgADP, MgATP, MgAMP-PNP, MgAMP + 2 mM vanadate, MgATP + 2 mM vanadate, MgATP + 2 mM vanadate + 1 μM Ko143, MgADP + 1 μM Ko143, MgATP + 1 μM Ko143 or 10 mM MgAMP-PNP + 1 μM Ko143. When Ko143 was present, the membranes were preincubated with this inhibitor for 5 min before the addition of other reagents.

5D3 binding is shown in the percent fluorescence measured in the presence of 5D3 alone (see Experimental procedures).

Values shown are means of at least four independent experiments ± standard deviation (S.D.) values.

Figure 1

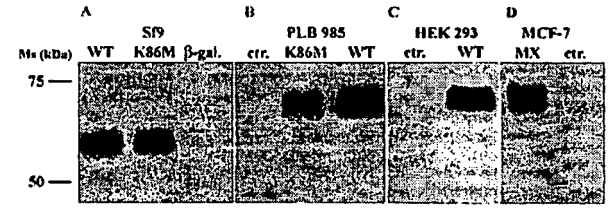


Figure 2

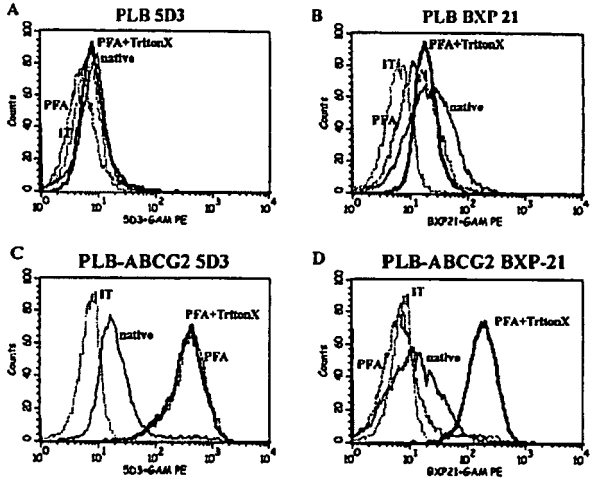


Figure 3

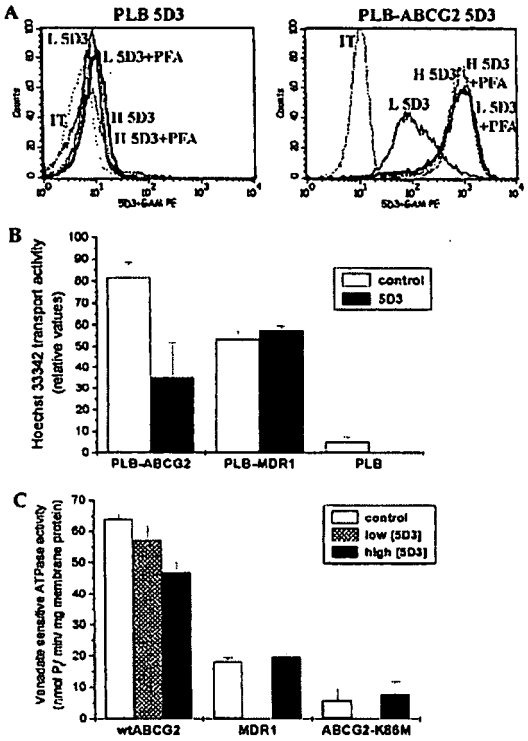


Figure 4

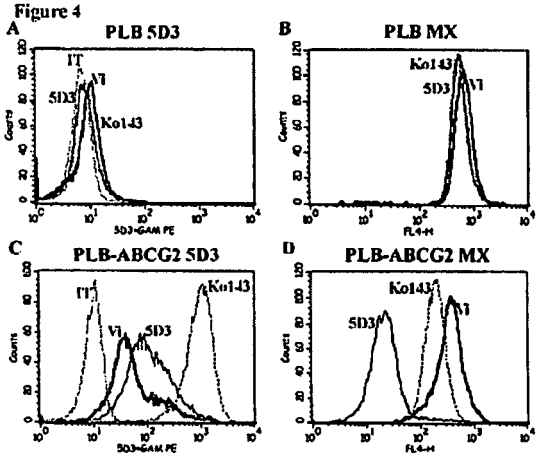


Figure 4

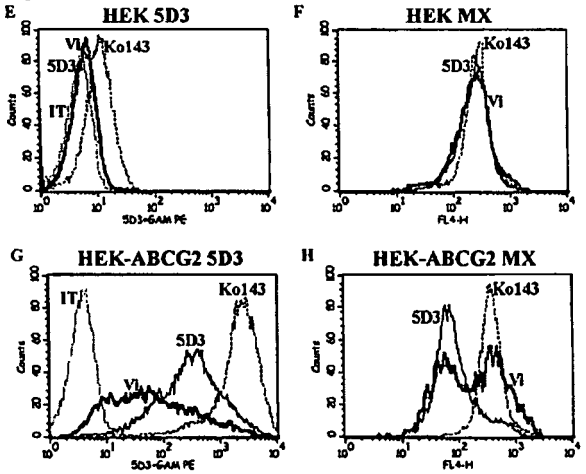


Figure 5

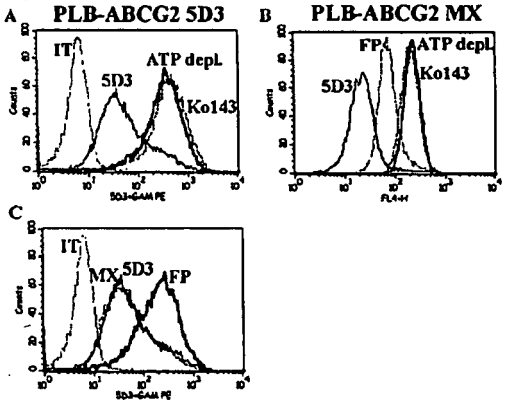


Figure 6

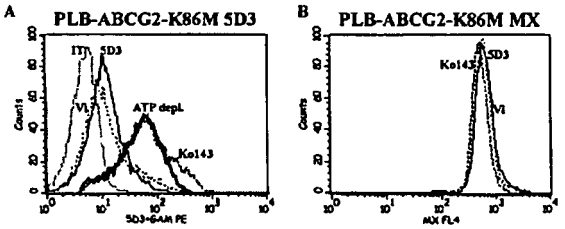


Figure 7